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COMPONENTS OF KRAFT MILL WASTE AND THEIR TOXICITIES

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Corvallis, Oregon

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Final Progress Report

Grant WP 00079

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FINAL PROGRESS REPORT WP-00079
COMPONENTS OF KRAFT MILL WASTE AND THEIR TOXICITIES

Historical

In the early 1950's an investigation of the influence of various fractions of kraft pulp mill wastes on the survival of silver salmon uncovered some significant new data on the toxicity of some fractions of this waste material. Studies by R. McHugh[1] indicated that an important fraction of the toxic material could not be removed by aeration of the waste, but was carried into the distillate during steam distillation. In 1958 a small grant was made by the Public Health Service to Charles Warren of the Fish and Game Department to enable a preliminary investigation of the feasibility of isolating and identifying the chemical constituents in the toxic steam distillate. From 1959-1961 work on this objective, along with a broader program of toxicity analysis of the various effluents from several kraft mills in Oregon having differences in engineering details and of waste handling procedures, was pursued vigorously under a grant with Dr. Warren as principal investigator and Elliot Marvell of the chemistry department as co-investigator. The results of these studies were treated in a previous progress report.[2]

PART A

THE CHEMICAL STUDIES

Introduction

In 1962 the major emphasis in this research program was shifted from the biological to the chemical aspects in order to proceed more expeditiously toward isolation and identification of some of the toxic substances in the steam distillable material from the mill wastes. Thus in February 1962, the present grant series WP-00079 was initiated with Dr. Marvell as principal investigator. The main aims of the grant were:

1. To separate in pure form and, where possible, identify the components of the evaporator foul condensate effluent of a selected kraft mill.
2. To synthesize when necessary components of the waste to provide a sufficient sample for biological study.
3. To determine the acute toxicity to fish of individual components of the evaporator condensate.
4. To determine the acute toxicity to mammals of individual components of the evaporator.
5. To provide appropriate groups within the National Institutes of Health samples of isolated and identified compounds for study of their chronic toxicity and carcinogenic activity in mammals.
6. To evaluate more fully the relative importance of readily separable groups of toxic constituents of whole mill effluents deriving from different sources within the mills, with attention to their persistence and interactions.

The present report will describe the work done and the results obtained primarily since February 1, 1962. Certain aspects of work done earlier will be included since they were not part of any earlier reports.

Personnel

The principal investigator, Elliot N. Marvell, was responsible for the chemical studies carried out during this grant, while the co-investigator, Charles E. Warren, provided guidance for the biological studies and supervised the bioassays. Only two further associates were involved in a significant way on this grant. Dr. Wolfgang Gänzler, a post-doctoral associate, was employed from October 1962 to July 1964, and Mr. Richard Banks has been hired as a half-time predoctoral research assistant from July 1964 until the grant terminated on June 30, 1967. A number of students were employed from time to time to carry out routine processing of wastes, but they did not contribute to the overall direction of the research effort.

Results of the Chemical Investigations

I. Source and Nature of the Mill Waste

The waste material used for the chemical studies was obtained exclusively from the Western Kraft Mill in Albany, Oregon. All samples were drawn from a single source within this plant, viz. the storage tank for the condensate from the low vacuum stage of the "weak black liquor" evaporator. This mill pulps exclusively chips of Douglas fir (Pseudotsuga menziesii) using a 2.5 hour cook at 170° with a liquor containing 7.5 lbs. of base (as sodium oxide) per cu. ft. and 25% sulfidity.[3] Only unbleached pulp is produced and the condensate which is derived from the low vacuum stage of the evaporation is normally used as hot water and wash water in the mill. The excess overflows to the sewer and constitutes a large portion of the mill effluent.

Generally collection of the condensate from the mill was carried out over a period of several weeks to eliminate, as far as is possible, the fluctuations in the character of the effluent due to small alterations in the mill practice. Length of storage of the chips, variations in washing of the pulp and changes in the operation of the evaporator are some of the factors which may influence the chemical content of the effluent. Samples were usually obtained in 20 liter Pyrex carboys and were processed as rapidly as possible after their receipt in the laboratory.

II. Recovery of Organic Constituents of the Waste

The original procedure used to recover the organic constituents in the "condensate" was based on the observation of McHugh[1] concerning the steam distillation of the toxic components. Thus the first batch of "condensate" was distilled under reduced pressure at 85-90°, and the organic materials

recovered from the distillate by extraction with ether.[4] The total organic material recovered generally was very consistent at the level of 0.125 g./l. Toxicity of the residue after distillation and of the aqueous layer after extraction of the distillate was reduced essentially to that of control samples obtained from an unpolluted creek. This procedure was not efficient from the point of view of the time required to process sufficient material to subject to further separation.

Accordingly when it became necessary to process further "condensate", we investigated alternative methods for the recovery of the toxicants. Extraction was the obvious alternative, but the "condensate" was found to contain enough dissolved salts capable of complexing diethyl ether to render impossible direct extraction with such basic solvents as ethers. Direct extraction with petroleum ether did prove feasible, but this non-polar solvent was not overly effective in removing the organic constituents of the waste. In fact only about 60% of the organic material, albeit a more highly toxic portion, can be economically removed in this way. The remainder of the material was recovered by saturating the aqueous layer remaining after the petroleum ether extraction with sodium sulfate and extracting with diethyl ether. The petroleum ether extract will be labelled PE in the report and the above ethyl ether extract will be labelled EE-1. A total of 120 l. of "condensate" was processed during this part of the work.

A final batch of "condensate" was obtained and its organic constituents removed. Again an attempt to simplify the extraction procedure was made. A continuous extractor employing methylene chloride as extraction solvent was designed and constructed. It was hoped that this solvent would prove sufficiently polar to remove all of the organic material in a single

extraction. Hand processing of small batches indicated that this was the case, but the lesser efficiency of the continuous process rendered complete recovery too slow and hence uneconomical. Thus once again the final recovery was made by saturating the aqueous residue with sodium sulfate and extracting this solution with ethyl ether. The ether extract will be abbreviated as EE-2 and the methylene chloride as MC.

Our further studies of the various extracts suggests that no very effective separation is achieved by the dual extraction scheme except at the extremes of polarity of the extracted substances. Thus for any future work we recommend that the "condensate" be saturated with sodium sulfate and submitted then to direct extraction with ethyl ether.

III. Column Chromatography

The work of Wiman,[3] which was reinforced by some subsequent observations of Gänzler described in this report, showed the futility of fractional distillation techniques as a preliminary separative method. Problems with decomposition, polymerization and interreactions of the components of the mixture proved that any substances isolated subsequent to such treatment would be subject to questions about their origin. Thus all of the work described in this report utilized column chromatography as the method of fractionation of the toxicants.

The general procedure employed used a large silica gel column which was wet packed under pentane. The silica gel was protected from atmospheric moisture during the entire operation in order to ensure that the main separative process involve adsorption rather than partition chromatography. The latter is very sensitive to temperature and no attempt was made to thermostat the column. A continuous gradation in solvent polarity was achieved

via a gradient elution and the eluant was collected in 40 ml. fractions by an automatic fraction collector. The solvent was removed from each fraction using a rotatory evaporator and aspirator vacuum. The fraction was weighed and finally a plot of the weight per fraction vs. fraction number was made. The fractions comprising single peaks on the chromatogram were combined and submitted to further study.

A. Separation of Extract PE.

Most of the extract PE (11.0 g.) was chromatographed on a column containing 750 g. of silica gel which had been activated at 140°C. Continuous gradation of the eluting solvent from pentane to chloroform and finally up to 50:50 chloroform/ethyl acetate was used. In all 790 fractions were collected and 85% of the material placed on the column was collected in the eluates. The plot indicated that the material eluted could be combined into twelve main divisions which will be labelled here PE-CC-1···PE-CC-12. Fractions PE-CC-1 through PE-CC-9 contained only 18% of the recovered material and no single fraction contained sufficient material to warrant further study at this state of the investigation. The last three fractions were submitted to toxicity analysis and the results are given in Table I. These three fractions were all studied further as is described in later sections.

Sample	Concentration in Mg/l		T_{Lm}
	in "condensate"	in sample	
Distilled water			86
Original "condensate"			15
PE			44
PE-CC-10	25	26.3	40
PE-CC-11	22	22.3	78
PE-CC-12	13	13.2	83

Table I. Bioassay results pertinent to the initial chromatographic separation of extract PE.

B. Separation of Extract MC

Fourteen grams of extract MC was subjected to column chromatography using 750 g. of BDH silica gel using a gradient elution progressing from 2% ethyl acetate in chloroform to 20% methanol in ethyl acetate. Of the 14 g. placed on the column 87% was recovered in 525 fractions. On the basis of information derived from the usual plot this was divided into twenty fractions. These are designated here as MC-CC-1···MC-CC-20. The results of the toxicity tests on these materials are shown in Table II.

C. Separation of Extracts EE-1 and EE-2

The ethyl ether extracts EE-1 and EE-2 were combined and the total material (21.6 g.) was chromatographed over 1000 g. of BDH silica gel. A gradient elution proceeding smoothly from 5% ethyl ether in pentane through mixtures of acetone in ethyl ether to methanol in ethyl ether and finally to pure methanol was carried out. A set of 584 fractions was collected and finally divided into twenty-two groups on the basis of the usual plot. Results of the bioassays on these fractions are shown in Table III. The fractions are labelled EE-CC-1···EE-CC-22. All of the material placed on the column was recovered.

Fraction	Weight of Fraction (g.)	Concentration in Test Sample (mg/l)	Kill* (24 hr.)
MC-CC-1	0.296	3.6	0/5
MC-CC-2	3.932	52.0	0/5
MC-CC-3	0.090	2.1	2/5
MC-CC-4	1.386	18.0	1/5
MC-CC-5	0.477	5.8	3/5
MC-CC-6	1.721	22.7	5/5
MC-CC-7	0.344	4.4	0/5
MC-CC-8	1.065	13.9	0/5
MC-CC-9	0.030		
MC-CC-10	0.092		
MC-CC-11	0.116	2.0	0/5
MC-CC-12	0.022		
MC-CC-13	0.043		
MC-CC-14	0.066		
MC-CC-15	0.128		
MC-CC-16	0.241	2.9	3/5
MC-CC-17	0.397	5.0	0/5
MC-CC-18	0.028		
MC-CC-19	0.026		
MC-CC-20	0.820	11.3	0/5
Crystals from MC-CC-15 + MC-CC-16	0.833	16.4	0/5

Table II. Bioassay data for the fractions separated from extract MC. *Data are given in number of fish killed per total exposed. The samples used were too small to permit accurate determination of T_{Lm} values.

Fraction	Weight (g.) in fraction	Concentration (mg/l) of Sample	Kill** (24 hrs.)
EE-CC-1	0.056		
EE-CC-2*	2.238		
EE-CC-3	0.187		
EE-CC-4	0.203		
EE-CC-5	0.727		
EE-CC-6	6.943	43.9	0/5
EE-CC-7	0.522	3.2	0/5
EE-CC-8	0.814	5.2	2/5
EE-CC-9	0.917	5.1	0/5
EE-CC-10	1.354	8.0	2/5
EE-CC-11	1.916	12.1	0/5
EE-CC-12	1.903	11.9	5/5
EE-CC-13	0.851	5.6	1/5
EE-CC-14	0.856	5.5	1/5
EE-CC-15	0.744	5.5	2/5
EE-CC-16	0.579	3.8	2/5
EE-CC-17	0.097		
EE-CC-18	0.237		
EE-CC-19	0.267		
EE-CC-20	0.237		
EE-CC-21	0.307		
EE-CC-22	0.488		

Table III. Bioassay data for fractions from extracts EE-1 and EE-2.

* Found to be Nujol. ** Data are given in number of fish killed per total exposed. Samples were too small to permit accurate determination of T_{Lm} values.

IV. Correlations Among the Fractions

One problem which has caused a certain amount of concern in this work has been the difficulty associated with correlating the various fractions obtained by various separation procedures from the "condensate". Obviously when pure or nearly pure substances were to be compared this provides no problem since standard spectral procedures are completely adequate. The difficulty appears where small fractions which are composed of several substances must be compared with other fractions, also mixtures, with different composition but possibly containing a single compound in common. While no single technique has been fully satisfactory, use of gas chromatography and thin layer chromatography combined in special cases with spectral measurements has been quite useful.

The following interrelations amongst the more toxic fractions were clearly ascertained by the indicated comparison methods. One of the common

Fractions		Common Components	Comparison
PE-CC-10	EE-CC-11	2	TLC
PE-CC-11	MC-CC-4	1	GPC + Deriv.
PE-CC-12	EE-CC-12	1	TLC
EE-CC-15	EE-CC-16 MC-CC-4	1	m.p.

constituents of PE-CC-10 and EE-CC-11 was apparently 4-(p-tolyl)-1-pentanol as indicated by comparison gas chromatography. This was clearly the common constituent in PE-CC-11 and MC-CC-4. Keto-alcohol B was the designation given the common substance to PE-CC-12 and EE-CC-12. The substance present in the three fractions listed last was cis-terpin hydrate.

V. Gas Chromatographic Analysis of Fractions

Although a number of fractions could not be passed through a gas chromatographic column without decomposition, much information about the composition of certain fractions was obtained and use of this data was made in deciding which fractions to study. The data obtained in this way are summarized in Tables IV and V. These data express clearly the grave difficulties faced in isolating pure materials from this complex mixture. Certain of the materials which have been identified or for which there is sufficient data to suggest a designation have been labelled in these tables. In most cases internal comparison was carried out, but in some cases the identity was derived from the retention time alone. Thus the indications of identity are not completely reliable and should be treated as suggestive rather than definitive.

Table IV. Summary of the gas chromatographic analysis of the fractions from the methylene chloride extract.

Fraction + Weight(g.)	Peak No.	%	Component	Conditions*
MC-CC-1	1	5		A
0.296 g.	2	10		
	3	60		
	4	1		
	5	5		
	6	5		
	7	4		
	8	5		
MC-CC-2	1	1		A
3.932 g.	2	1		
	3	1		
	4	0.5		
	5	0.5		
	6	80	guaiacol	
	7	3		
	8	15	4-ethyl-2-methoxy phenol	
MC-CC-3	1	30		A
0.090 g.	2	5		
	3	40	guaiacol	
	4	20		

Fraction + Weight(g.)	Peak No.	%	Component	Conditions
MC-CC-4	1	0.5		A
1.386 g.	2	0.1		
	3	1		
	4	1		
	5	15	α-terpineol	
	6	2		
	7	3		
	8	2		
	9	78	4-(p-tolyl)-1-pentanol	
MC-CC-5	1	18		A
0.477 g.	2	5	terpineol	
	3	1		
	4	15		
	5	5		
	6	5		
	7	1		
	8	5		
	9	4		
	10	30	40(p-tolyl)-1-pentanol	
MC-CC-6	1	1		B
1.721 g.	2	2		
	3	0.5		
	4	2	4-(p-tolyl)-1-pentanol	
	5	1		
	6	90	keto-alcohol B	
	7	5		
MC-CC-7	1	2		B
0.344 g.	2	2	terpineol	
	3	2		
	4	5		
	5	1		
	6	1		
	7	0.5		
	8	1	4-(p-tolyl)-1-pentanol	
	9	1		
	10	2		
	11	30		
	12	60	keto-alcohol B	
MC-CC-8	1	12		B
1.065 g.	2	10		
	3	5		
	4	12		
	5	2		
	6	5		
	7	55		
	8	1		
	9	1		
	10	1		
	11	1		
	12	1		
	13	1		

Fraction + Weight(g.)	Peak No.	%	Component	Conditions
MC-CC-9 0.030 g.	not run			
MC-CC-10	1	45		B
0.092 g.	2	5		
	3	50		
MC-CC-11	1	7		B
0.116 g.	2	5		
	3	5		
	4	50		
	5	5		
	6	5		
	7	7		
	8	15		
MC-CC-12 0.022 g.	not run			
MC-CC-13	1	5		B
0.043 g.	2	80		
	3	5		
	4	10		
MC-CC-14 0.066 g.	not run			
MC-CC-15	1	1		B
0.128 g.	2	1		
	3	90	terpin hydrate	
	4	7		
	5	1		
MC-CC-16	1	2		B
0.241 g.	2	95	cis-terpin hydrate	
	3	2		
	4	1		
MC-CC-17	1	2		B
0.397 g.	2	35		
	3	3		
	4	60		

*Conditions: A 15 ft. x 1/4 in. 5% FFAP on 40/60 chromosorb G at 200°
B 5 ft. x 3/8 in. 5% FFAP on 45/60 chromosorb G at 200°

Table V. Summary of the gas chromatographic analysis of the fractions from the ethyl ether extracts.

Fraction + Weight(g.)	Peak No.	%	Component	Conditions
EE-CC-1	1	5		A
0.056 g.	2	3		
	3	3		
	4	5		
	5	5		
	6	5		
	7	3		
	8	10		
	9	15	guaiacol	
	10	15		
	11	15		
	12	15		
EE-CC-2	mainly Nujol			
2.238 g.				
EE-CC-3	1-7	0.5		A
0.187 g.	8	1		
	9	3		
	10	5		
	11	1		
	12	2		
	13	0.5		
	14	3	guaiacol	
	15	2		
	16	1		
	17	40	4-ethyl-2-methoxyphenol	
	18	20		
	19	15		
	20	1		
	21	2		
	22	3		
EE-CC-4	1	5	4-ethyl-2-methoxyphenol	A
0.203 g.	2	10		
	3	30		
	4	10		
	5	15		
	6	30		
EE-CC-5	1	1		A
0.727 g.	2	1		
	3	2		
	4	4		
	5	30		
	6	2		
	7	20		
	8	15		
	9	10		
	10	2		
	11	4		
	12	4		

Fraction + Weight(g.)	Peak No.	%	Component	Conditions
EE-CC-6	1	1		B
6.943 g.	2	1.5		
	3	2		
	4	2		
	5	1		
	6	0.5		
	7	2		
	8	90	1720 ketone	
EE-CC-7	1	2		B
0.522 g.	2	2		
	3	2		
	4	2		
	5	40		
	6	2		
	7	50	1720 ketone	
EE-CC-8	1	3		B
0.814 g.	2	3		
	3	40		
	4	2		
	5	12		
	6	5		
	7	10		
	8	5		
	9	12		
	10	8		
EE-CC-9	1	0.5		B
0.917 g.	2	0.5		
	3	90		
	4	2		
	5	2		
	6	1.5		
	7	1.5		
	8	1.5		
	9	0.5		
EE-CC-10	1	1		B
1.354 g.	2	19		
	3	5		
	4	2		
	5	2		
	6	1		
	7	2		
	8	25		
	9	18		
	10	25		
EE-CC-11	1	1.5		B
1.916 g.	2	0.5		
	3	1		
	4	4		
	5	2		
	6	1		
	7	5		

Fraction + Weight(g.)	Peak No.	%	Component	Conditions
	8	15		
	9	70	4-(p-tolyl)-1-pentanol	
EE-CC-12	1	1		B
1.903 g.	2	1		
	3	5		
	4	2		
	5	1		
	6	20		
	7	35		
	8	35	keto-alcohol B	
EE-CC-13	1	20		B
0.851 g.	2	20		
	3	10		
	4	20		
	5	5		
	6	5		
	7	20		
	8	trace		
EE-CC-14	1	40		B
0.856 g.	2	25		
	3	10		
	4	5		
	5	10		
	6	2		
	7	2		
	8	1		
	9	5		
EE-CC-15	1	60		B
0.744 g.	2	25		
	3	2		
	4	2		
	5	2		
	6	2		
	7	3		
	8	2		
	9	2		

*Conditions A 15 ft. x 1/4 in. 5% FFAP on 40/60 chromosorb G at 200°
B 5 ft. x 3/8 in. 5% FFAP on 45/60 chromosorb G at 200°

VI. Structure Identification Studies

Owing to the complexity of the mixture and the large number of fractions separated from the condensate, it was necessary to decide which fractions should be given attention first. Two criteria were applied in making this judgement. The main consideration was, quite naturally, the toxicity of the fraction. A subsidiary criterion was the amount of material present as a single component in the fraction. To initiate work with an amount clearly insufficient to permit a full structural study would be a waste of valuable time.

A. Gas Chromatographic Comparison Studies.

A few volatile substances among the very large number of trace components of the "condensate" have been tentatively identified by gas chromatographic comparison with known samples. Cross checking of those initially identified on a Carbowax 20 M column by carrying out a second study of the same material on a Craig polyester column led to final identification of four compounds, limonene, terpinene, p-cymene and anisole. The details have been published,[4] and will be available to readers of this report since a reprint of the paper is included in this document.

B. 4-(p-Tolyl)-1-pentanol.

The main discovery stemming from work done mostly on the grant preceding the present one was the identification of S(+) 4-(p-tolyl)-1-pentanol in the "condensate". This substance, clearly derived from a sesquiterpene, was identified by standard methods and its identity confirmed by synthesis. This was reported in a paper by Marvell and Wiman,[4] and a reprint is included in the appendix to this report. Though relatively active biologically, 4-(p-tolyl)-1-pentanol was not responsible for any large fraction of the total toxicity of the condensate.

More recent work has shown that this alcohol is always present in relatively large amount in all samples of the "condensate" we have examined. Fraction PE-CC-11 was identified by its spectral properties as 4-(p-tolyl)-1-pentanol and the identification confirmed by preparation of a 3,5-dinitrobenzoate, m.p. 76.5-77°. This fraction constitues 23% of the total petroleum ether extracts. Similarly, MC-CC-4, which comprises 10% of the methylene chloride extract, was identified as the same alcohol.

C. Guaiacol and 4-Ethyl-2-methoxyphenol.

Guaiacol is one of the main products of the thermal decomposition of Douglas fir lignin.[5] It is found as expected in the "condensate". The fraction PE-CC-10, a very sharp peak on the column chromatography plot, contained about 25% of the total petroleum ether extract. Gas chromatographic examination on a Carbowax 1500 column indicated that it consisted of a single substance which appeared to be guaiacol on the basis of internal comparison. However the infrared spectrum of the whole fraction showed a carbonyl band at 1700 cm^{-1}. Distillation of the crude fraction under reduced pressure gave pure guaiacol, b.p. 130° (15 mm.), which formed a p-nitrobenzoate, m.p. 99-102°. This fraction made up about 35% of the total PE-CC-10. A small amount of a yellow oil distilled at a higher temperature and will be considered below under the title hydroxy-ketone A. The main portion of PE-CC-10 decomposed during the distillation and gave rise to a viscous, presumably polymeric, residue. No further study was made of this residual material.

Fraction MC-CC-2 contained 3.93 g. or about 30% of the recovered material from the methylene chloride extract. Gas chromatography on an FFAP (Varian - Aerograph designation for their "free fatty acid phase") column showed that guaiacol constituted about 95% of this fraction. Comparison gas chromatography,

preparative gas chromatographic separation and the spectra of the pure compound confirmed this analysis. The minor (5%) component was also isolated from the preparative chromatography. Its n.m.r. spectrum showed the following peaks; triplet (3H) J = 7.3 cps at 1.20 ppm, quartet (2H) J = 7.3 cps at 2.54 ppm, singlet (3H) at 3.83 ppm, singlet (1H) at 5.2 ppm, a broad multiplet centered near 6.58 ppm and a singlet at 6.67 ppm. The multiplet and singlet together integrate for three protons. The infrared spectrum of this fraction shows bands at 3480 broad (OH), 3020, 2960, 2920, 2850, 1600, 1510, 1370, 1270, 1235, 1152, 1125, 1035, 850 and 805 $cm.^{-1}$. These data suggest that the substance is 4-ethyl-2-methoxyphenol. The small amount of material recovered from the chromatographic separation precluded a more complete identification. Since the entire fraction MC-CC-2 did not contribute in any measurable way to the toxicity of the condensate, we did not consider it necessary to synthesize a comparison sample.

D. cis-Terpin Hydrate (p-Menthane-1,8-diol).

The first ether extract, EE-1, deposited a set of white crystals, m.p. 116-118°. The n.m.r. spectrum showed peaks at 1.2 and 1.5 ppm, both multiplets having approximately equal areas under the peaks. The spectrum was run as a relatively dilute solution in deuterochloroform. The infrared spectrum showed bands at 3535 (OH), 3410 broad (H-bonded OH), 2925, 2890, 2820, 1460, 1448, 1370, 1175, 1105, 991, and 903. Neither spectrum shows any bands attributable to unsaturation. The number of methyl groups and the presence of tertiary hydroxyl suggests this is of terpene origin. The melting point suggested that this substance was cis-terpin hydrate, and the assignment was confirmed by spectral comparison with an authentic sample. This glycol is normally formed by addition of water to α-pinene under acidic conditions,

and it is not considered to be of natural occurrence.[6]

The glycol also appears in fractions MC-CC-15 and MC-CC-16. It separates as a crystalline precipitate from these fractions on standing. It constitutes about 10% of the total methylene chloride extract. At the concentration level present in the condensate cis-terpin hydrate exhibits no observable toxicity to fish.

E. Trans-Sylveterpin (m-Menthane-1,8-diol)?

A second solid alcohol, m.p. 139-141°, was isolated from fraction MC-CC-10. Crystalline material weighed only 43 mg. so that only spectral data were obtained. Both the infrared and n.m.r. spectra were very similar to those of cis-terpin hydrate so that we assume this is a closely related diol derived from some terpene present in the Douglas fir. The melting point suggests this is trans-sylveterpin, a diol derived from sylveterpineol,[7] and ultimately probably from the naturally occurring Δ^3-carene.[8] No sample was available for comparison, so a small amount was synthesized from Δ^3-carene by the method of Perkin and Tattersall.[9] This gives mainly the cis-isomer, but the trans-isomer is also present as a lesser component of the product. Gas chromatographic comparison showed that the alcohol isolated corresponded to the minor product in the synthesis. However the synthetic product was obtained in low yield and in impure form. Since this synthetic material was not adequately characterized, we consider the present structural assignment to be only a tentative one. The alcohol is only a minor component in the "condensate" and does not contribute to the toxicity to fish.

F. α-Terpineol (Δ^1-p-Menthen-8-ol).

The presence of cis-terpin hydrate suggested that either α-pinene or α-terpineol might also be present in the "condensate". Though α-pinene was not identified with certainty, the presence of α-terpineol in MC-CC-4 was indicated by internal gas chromatographic comparison with the authentic substance.

This substance was only a minor component in the "condensate" and did not contribute significantly to its toxicity to fish.

G. Keto-Alcohol A

Fraction PE-CC-10 was found to contain substantial amounts of guaiacol, contaminated by at least a second substance having a carbonyl band in the infrared at 1700 $cm.^{-1}$. When an attempt was made to separate the two substances by distillation from an alembic under reduced pressure, the guaiacol was obtained in relatively pure form, b.p. ca 130° (15 mm.). A high boiling slightly yellow oil, 1.0 g., b.p. 125-145° (0.11 mm.), appeared to contain the ketone. A considerable amount of polymeric material remained in the still pot. It is not clear whether the ketone apparently present in the yellow oil is identical with that originally present in the mixture, or whether some alterations have taken place during the distillation. This high boiling oil was found to exhibit a high degree of toxicity to fish, but no similar product has been isolated from the "condensate" in subsequent studies.

The high boiling liquid gives an infrared spectrum showing bands at 3400 $cm.^{-1}$ broad (OH), 1700 (carbonyl), 1450, 1360 ($C-CH_3$), 1250 (C=O?), 1115, 1065 and 740 $cm.^{-1}$. Contamination by guaiacol was still evident. The compound was designated keto-alcohol A. It did not form a solid 2,4-dinitrophenylhydrazone. In view of the uncertain origin of this compound, and its impure form, further investigation of its structure was postponed.

H. Keto-Alcohol B.

Fraction PE-CC-12, representing 12% of the petroleum ether extract, appeared to be a single substance. Gas chromatographic analysis showed the presence of a series of trace contaminants, but a minimum of 91% of the

fraction appeared under a single peak in the gas chromatogram. This major component was not separable by any thin layer chromatographic method tried. Use was made of silica gel, aluminum oxide, and magnesium silicate, each with a variety of developing solvents. Subsequently, what appears to be the same compound (gas chromatographic analysis and similarity of infrared spectra) was found in fractions EE-CC-12 and MC-CC-6 and MC-CC-7. The toxicity data, however, were contradictory since PE-CC-12 was of relatively low toxicity toward fish while by EE-CC-12 and MC-CC-6 exhibited a high degree of toxicity. Since none of the samples used for these tests was pure, the data may not mean much vis-a-vis the toxicity of the pure keto-alcohol B.

The sample of keto-alcohol B isolated from PE-CC-12 was optically active, $[\alpha]_D^{25}$ + 12.5° ($CHCl_3$), and was obtained as a clear liquid. Its infrared spectrum showed bands at 3420 broad (OH), 3025 (C=CH), 2940, 2870, 1710 (saturated ketone), 1470, 1455, 1412 (CH_2-$\overset{O}{\overset{\|}{C}}$?), 1380 sh., 1370 (C-$CH_3$ or possibly $C(CH_3)_2$), 1170, 1145 both weak, 1054, 1035, 957 weak, 930 and 895. Its ultraviolet spectrum had two maxima, λ_{max} 283 (E=80, based on 238 as molecular weight), λ_{max} 200 (E=3000). The first peak is due to a saturated carbonyl but the extinction coefficient is probably too high. It could only be estimated since the tail of the more intense peak at 200 mμ overlapped this region. The 200 mμ peak appears to indicate the presence of a double bond, perhaps trisubstituted, but the extinction is too low and this may be an impurity (see below). The n.m.r. spectrum of a solution in carbon tetrachloride exhibited the following series of peaks; doublet at 3.46 ppm (J = 6.5 cps), singlet at 2.49 ppm which moved to 2.06 ppm when the sample was diluted, multiplet centered at 2.19 ppm, a broad series of peaks between 1.1 and 1.9 ppm, and three peaks between 0.81 and 1.0 ppm. There was no resonance in the olefinic proton region!

A very comprehensive investigation of the derivatives of keto-alcohol B was made. It will form under normal conditions a phenylhydrazone, a 2,4-dinitrophenylhydrazone, an oxime, a semicarbazone, an acetate, a benzoate, a p-nitrobenzoate, a 3,5-dinitrobenzoate, a phenylurethane and an α-naphthylurethane. None of these was a solid material! This fact seemed to throw a reasonable doubt about the purity of the keto-alcohol B. Interestingly enough, however, a thorough examination of each derivative by a variety of thin layer chromatographic techniques failed to separate any of these. Thus to all tests we were able to apply up to this point the keto-alcohol seemed to be a single compound. At this point access to a mass spectrometer became available and a mass spectrum of the keto-alcohol B isolated from PE-CC-12 was obtained. This showed a series of parallel mass peaks separated by two mass units. Since regular loss of a hydrogen molecule from a series of ions seemed unusual, this seemed to indicate that a mixture of two similar molecules, one having one more point of unsaturation than the other, was in hand. The ultraviolet spectral data appear to confirm this assumption. A great deal of effort was expended in trying then to separate this mixture. The material was oxidized and derivatives formed and their separation attempted. Similarly the molecule was reduced with lithium aluminum hydride and the diol derivatives prepared. These also failed to separate. The mixture was submitted to catalytic hydrogenation, but this led to no alteration in the properties.

The mass spectrum showed a peak at 222 as the highest mass number. This may not be the molecular ion since alcohols are particularly prone to lose water.[10] If the proper molecular weight is 240, the molecule has the formula $C_{15}H_{28}O_2$. Unfortunately, the integration of the n.m.r. spectrum does not lead to a count for 28 protons if the hydroxyl peak is considered to

be a single proton. Such a count would suggest that 18 protons were present. Note however the discussion below under the acetate. However the presence of peaks separated by two mass units and the ultraviolet spectrum suggested very strongly that this keto-alcohol was contaminated by an unsaturated molecule of great similarity.

The keto-alcohol B isolated from EE-CC-12 was converted to a monoacetate and purified by gas chromatography. The infrared spectrum of this acetate showed peaks at 2960, 2920 and 2870 (aliphatic CH), 1740 ($OCOCH_3$), 1720 (ketone), 1465, 1455, 1365 ($C-CH_3$), 1235, and 1030. The n.m.r. spectrum showed the following pattern; doublet at 3.96 ppm (J = 7.0 cps), multiplet between 2.1 and 2.4 ppm, singlet at 1.97 ppm, another multiplet between 1.1 and 1.8 ppm and a series of sharp peaks between 0.8 and 1.0 ppm. If it is assumed that the doublet at 3.96 represents the CH proton of a CHOAc group, then the proton count for the acetate is 24. If, on the other hand, the singlet at 1.97 which represents the acetate methyl group is used as an internal standard, the proton count reaches 28. Finally, a proton count of 30 is obtained when the series of peaks between 0.8 and 1.0 ppm is assigned to three methyl groups or nine protons. There are no olefin proton peaks present in the n.m.r spectrum, and examination of the acetate by the tetranitromethane method,[11] confirmed the absence of any double bonds.

The mass spectrum of the acetate (see Table VI) indicated that it was reasonably pure since a series of scans taken during its diffusion into the ionization chamber showed that the diffusion produced no fractionation. The molecular ion peak was at 282 and was accompanied, as expected for an acetate, by an M^+ -60 peak at 222. Two fragments which apparently involve loss of a methyl radical appear at 267 and 207. Consequently the acetate can be assigned

Table VI. Comparison of Mass Spectral Data for the Keto-Alcohol B and Its Acetate.

	Rel. Abund.			Rel. Abund.	
m/e	OH	OAc	m/e	OH	OAc
282		1.7	69	40	38.9
222	2.1	5.3	67	75	36.8
220	2.5	1.8	65	50	9.2
			63	22	3.0
207		2.8			
204		9.7	58		15.6
163	33	8.8	57	99	75.5
161	6	8.8	56		16.7
			55	86	42.2
147		17.7	53	69	17.3
			51	43	8.0
132	31	2.4			
127		61.2	45		5.5
120	29	25.2	44		22.7
119	80	35.4	43	86	60.8
117	31	4.9	42	46	54.7
115	26	4.7	41	93	92.1
			40	30	8.7
107	40	30.2	39	88	29.9
105	47	28.9			
95	26	31.1	31		3.8
94		21.5	30		1.6
93	76	63.6	29	85	39.9
92		12.6	28	28	100
91	100	26.9	27	91	29.6
85	29	41.6			
83		14.2			
82		8.3			
81	51	40.7			
80		10.5			
79	99	37.5			
78	26	9.2			
77	85	22.3			
73		7.6			
72		2.7			
71		16.3			

the formula $C_{17}H_{30}O_2$, and the keto-alcohol B has the formula $C_{15}H_{28}O_2$. The molecule is thus derived from a monocyclic sesquiterpene. This suggests perhaps that this substance is derived from the same sesquiterpene as is the 4-(p-tolyl)-1-pentanol. One point which detracts from this idea is that it would necessitate the presence of four methyl groups and the n.m.r. data will not admit of more than three.

The n.m.r. spectra of both the alcohol and the acetate show great similarity in the C-Me region (0.8-1.0 ppm) and in the 2.1-2.4 region. The former area shows at least four bands in each spectrum. No band appears to be large enough and sharp enough to constitute a singlet (compared with the singlet at 1.97 in the acetate, for example). Apparently then all methyl groups are adjacent to a carbon bearing at least a single proton. The 2.1-2.4 region can be assigned to protons on carbons adjacent to the carbonyl group. The integration is not accurate enough to judge whether there are three or four such alpha protons. Up to now no sample has been pure enough to warrant deuturation to clarify this point, but this experiment is now in progress. The splitting pattern in this region is difficult to discern and permits several possible interpretations.

Work is now underway to attempt to ascertain the nature of the carbon skeleton. The keto-alcohol B can be reduced conveniently with sodium borohydride to give two diols, probably epimeric. These are obtained in about a 10:1 ratio. An impure unsaturated alcohol has also been obtained. The major diol corresponds to a minor peak in the gas chromatogram of EE-CC-12. The diol shows a very high toxicity, being far and away the most toxic substance to fish we have yet obtained.

I. The 1720 Ketone:

Fraction EE-CC-6, the largest single fraction in the chromatographic separation of the ether extracts, was analyzed by gas chromatography on a 5% FFAP column. This showed the presence of six components in the crude fraction, but the slowest to elute constituted ca. 90% of the total. The material was separated by preparative gas chromatography and the major component isolated in a form which was uniform to glc. Since it made no important contribution to the total toxicity of the ether extract this fraction was not carefully investigated. However, the normal spectral data were obtained, and are discussed below.

The infrared spectrum showed bands at 3060 (arom. H), 3010 (O-CH_2-O?), 2960, 2920, 2850 (aliph. or alicyclic CH), 1720 (carbonyl, probably ketone), 1600, 1580 (arom. ring), 1460, 1450 (CH_2), 1380, 1360 (CH_3- and $(CH_3)_2$-CH-), 1275 very broad (C=O, C=C-O-C ?), 1125 strong, 1070, 1040 (C-O-C, aromatic ?), 785, 760 sh., 745 sh. (arom. out-of-plane bending). The ultraviolet spectrum showed λ_{max} 272 (ϵ = 320), 222 (ϵ = 1700), which tends to confirm the substituted aromatic moiety.[12] The extinction coefficient of the short wavelength band appears to be unexpectedly low, but the extinction coefficients which were based on a molecular weight (osmometer) of 320 are subject to revision. The n.m.r. spectrum shows an apparent A_2B_2 pattern centered at 7.54 ppm (aromatic protons), two singlets at 4.19 and 4.11 ppm respectively, a broad multiplet centered at 1.97 ppm and two large multiplets between 1.7 and 0.9 ppm. The integration shows relative areas for the four groups of peaks of 2:2:1:15.

The mass spectrum shows most significantly that this ketone is not pure. A pair of scans at different times during the diffusion of the ketone into the ionization chamber differ dramatically. The earlier scan which is reproduced in Table VII has no peak higher than 192 while the later scan shows a number of small peaks in the 300-500 range. The substance is apparently optically active showing a plain ORD curve. At 320 mμ the apparent activity is $[\alpha] = +8.96^{\circ}$. This could, of course, be due to an impurity.

These data do not define the structure of this ketone, but several significant points can be noted. This is an aromatic compound, probably para disubstituted, but not having any methyl groups on the ring. Thus it does not appear to be related to 4-(p-tolyl)-1-pentanol. It apparently contains more than a single oxygen atom, all beyond the ketone oxygen being ether types. However, none of these are methyl ethers since no CH_3-O- protons appear in the n.m.r. spectrum. If the protons responsible for the singlets at 4.11 and 4.19 are on the same carbon, O-CH_2-CH group, the coupling in the doublet, J = 5 cps, would be unlikely for an aliphatic chain. Further experiments aimed at purification and identification of this compound are underway.

Table VII. Mass Spectral Data for MC-CC-6*

m/e	Rel. Abund.	m/e	Rel. Abund.
192	.6	85	1.5
167	.2	84	3.7
166	.3	83	2.0
165	.4	82	5.9
164	.3	81	3.9
163	.4	80	5.8
162	3.5	79	6.6
161	.5	78	2.6
160	.8	77	3.8
149	1.4	72	1.2
147	6.2	69	1.3
145	4.6	67	2.1
135	.7	66	1.4
134	.6	65	2.4
133	2.3	63	1.5
132	1.3	59	1.3
131	1.3	58	28.7
129	1.0	57	2.1
128	1.4	55	3.2
127	.7	53	2.6
123	.9	52	1.4
121	6.2	51	2.9
119	21.3	50	1.3
117	22.9	49	2.3
115	1.8	47	2.5
107	3.0	44	6.6
105	2.0	43	100
104	.9	42	7.6
103	1.3	41	6.8
96	3.9	40	2.1
95	1.2	32	19.4
94	1.7	31	29.1
93	3.5	29	20.6
92	4.0	28	52.9
91	4.8		

* m/e of rel. abund. less than 0.5 omitted below 160 and less than 1.0 omitted below 100.

XII. Publication Plans.

If the two important compounds, keto-alcohol B and the 1720 ketone, can be identified these results will be submitted to the Journal of Organic Chemistry for publication. If these cannot be completely identified with the material on hand the description of the work done will be submitted to a journal such as TAPPI where the results will be available to those who must pursue further studies in this area.

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IX. Summary.

The condensable material from the low vacuum stage of the evaporator in a typical kraft pulp mill pulping exclusively Douglas fir has been examined in an attempt to determine the nature of the compounds responsible for its toxicity to fish. These organic substances in the "condensate" were recovered by extraction procedures. The extracts were carefully separated into fractions by column chromatography using gradient elution techniques. Further analysis and purification of the fractions obtained from the column chromatography was made by gas chromatography and by thin layer chromatography. Biological assay of the fractions was carried out using guppies as the test organism.

The materials separated from the "condensate" were examined spectroscopically and chemically. Whenever possible the substances tentatively identified in this manner were compared with authentic samples, which were obtained via synthesis if not available commercially. In this way eight compounds were clearly identified. These were limonene, terpinene, p-cymene, anisole, guaiacol, cis-terpin hydrate, α-terpineol, and 4-(p-tolyl)-1-pentanol. Two others were identified tentatively as 4-ethyl-2-methoxyphenol and trans-sylveterpin. Three further substances were separated and examined. These were identified as keto-alcohols A and B and the 1720 ketone. Keto-alcohol A may be an artifact and was identified only in a single extract. The other two unknown components have been found repeatedly and are being studied further.

PART B

THE BIOLOGICAL STUDIES

INTRODUCTION

This part of the progress report covers all research on the toxicity of kraft pulp mill wastes to fish which has not been reported earlier. Some of the results from earlier incomplete experiments already reported are also included here in order to bring all of the completed data together for evaluation. Experiments on the relative toxicity to guppies of fractions of whole effluents from the three kraft mills were finished and all of the data examined. For the most part, additional data verify earlier findings, and it has not been necessary to change any previously stated conclusions.

Experiments to show the relative toxicity of whole wastes to several species of fish, and to show the importance of highly volatile and rapidly oxidizable toxic substances in the wastes were also run.

Many of the same methods and experimental conditions were used throughout the entire study period, and there has been no need to revise many of the descriptions used in previous reports.

METHODS AND MATERIALS

The fish used as test animals were guppies, Poecilia reticulata, bluegills, Lepomis macrochirus, coho salmon, Oncorhynchus kisutch, largemouth bass, Micropterus salmoides, threespine sticklebacks, Gasterosteus aculeatus, and goldfish, Carassius auratus. The guppies were reared in the laboratory under constant environmental conditions, and the other fish were obtained from ponds or streams near Corvallis, Oregon, from a nearby salmon hatchery, or from tropical fish dealers. Before any fish were used in experiments they were acclimated to laboratory conditions.

The toxicity bioassay methods used were those described by Doudoroff et al. ("Bio-assay Methods for the Evaluation of Acute Toxicity of Industrial Wastes to Fish." Sewage and Industrial Wastes, Vol. 23, pp. 1380-97, 1951), with some modifications. All tests were performed at 20° C in a constant-temperature room. The wastes and waste fractions tested were diluted with natural stream water. Median tolerance limits (TL_m), or those concentrations of tested wastes in per cent by volume that are lethal to 50 per cent of the test animals within specified exposure periods, were estimated by graphical interpolation after survival percentages at different tested concentrations of a logarithmic series had been observed.

In all routine tests only guppies 10 to 20 days old were used, and ten fish were exposed to each tested concentration or dilution of waste in a 1-liter Pyrex glass beaker containing 1 liter of the medium. Each dilution was continually oxygenated (aerated) by releasing a stream of air bubbles (about 500 per minute) from a small glass tube immersed in the medium, so as to maintain uniformly high dissolved oxygen concentrations.

Toxicity of whole wastes to several species of fish

Comprehensive tests on the relative tolerance of different species of fish to whole mill wastes were performed from February through April, 1961. The fish used in these tests were coho salmon 35 to 55 mm, bluegills 35 to 50 mm, both age-class zero; mature threespine sticklebacks 35 to 50 mm; small goldfish 70 to 90 mm; 10- to 20-day-old guppies 11 to 13 mm; and mature guppies 25 to 30 mm. Five gold fish and mature guppies, and ten coho salmon, bluegills, threespine sticklebacks, and young guppies were tested together in 36 liters at each waste concentration and control.

Toxicity of highly volatile and rapidly oxidizable substances

A series of bioassays was run to determine the contribution of highly volatile and rapidly oxidizable substances in whole wastes from all three mills to the overall toxicity. These tests were performed at irregular times from April 1960 through July 1963. All test conditions were similar to those described above for young guppies, with the exception that different amounts of oxygenation (aeration) were supplied to the pulp waste samples and bioassay solutions. The wastes were used in these bioassays either untreated as they were collected from the mill) or vigorously aerated for 4 to 24 hours to maintain a high oxygen concentration and to drive off the readily volatile compounds. The bioassays on these wastes were performed either without oxygenation, with very slow oxygenation, or with normal aeration (500 bubbles per minute),

Toxicity of distillates and residues from distilled whole wastes

Grab samples of whole effluents from Mills A, B and C were collected as nedded. A 4-liter portion of each sample was aerated for 4 hours before being

distilled, leaving only a small volume of undistilled residue (about 3 per cent of initial volume). One-half of the residue and of the distillate from each sample were diluted with water so that the volume of each dilution would be equal to one-half of the volume of the distilled sample portion. The remaining half of the residue was recombined with the other half of the distillate. Toxicity bioassays of the diluted residues and distillates, and of the residues recombined with distillates from the same samples, were performed with guppies 10 to 20 days old. Bioassays of fresh sample portions of wastes that had only been aerated for 4 hours, of untreated portions, and usually of portions that had been aerated for 24 hours, were performed also.

In order to facilitate comparison and interpretation of the test results, 24-hour, 48-hour, and 96-hour "normalized toxicity indices" were computed for each material tested. The normalized toxicity index for any sample portion or fraction is equal to the TL_m of that sample portion which had been aerated for 4 hours divided by the corresponding TL_m value of the sample portion or fraction in question. Thus, a toxicity index value of 1.00 was assigned to each sample portion that had only been aerated for 4 hours. A normalized toxicity index value of 0.50 signifies that the sample portion or fraction to which it applies was apparently only one-half as effective (as a toxicant) within the specified exposure period as the portion that had been aerated for 4 hours. The latter material had to be diluted twice as much as the former in order to obtain 50 per cent survival of the test animals for the specified exposure period. A normalized toxicity index value of 1.00 signifies no difference in effectiveness between the sample portion under consideration and that which had been aerated for 4 hours; and an index value of 2.00 would indicate toxicity apparently just twice as great as that of the sample portion aerated for 4 hours.

RESULTS AND DISCUSSION

Because of differences of mill design and operation, and of waste-disposal facilities, kraft mill wastes that are considered herein as being essentially similar in nature are by no means strictly comparable. For example, "whole mill effluents" from Mills A and B include essentially all waste waters from pulping and paper-making operations discharged to streams through common sewers. The so-called "whole mill effluent" of Mill C, on the other hand, does not include a large volume of waste water that is considered to be relatively harmless and is disposed of through a separate sewer system and waste outfall. Dilution of the more toxic effluent with this water doubtless would result in a decrease of toxicity. Even when true whole effluents only are considered, differences of their relative toxicities obviously cannot be taken to reflect accurately differences in quantities of toxicants discharged to receiving waters per ton of pulp or paper produced, inasmuch as volumes of waste water discharged per ton of product are not uniform. As might be expected, much variation was found in toxicity of different whole waste samples and in samples of waste components from the same mill.

In tests with whole effluent samples from all three mills five species of fish were tested together to compare their relative tolerance to the different wastes. The normalized toxicity index has been found for each species of fish by dividing the 96-hour TL_m for each group tested. This always gave young guppies a normalized toxicity index of 1.00. Any group of fish which had a normalized toxicity index greater than 1.00 was less tolerant than young guppies to the toxicant tested, any any species or group which had a normalized toxicity index less than one was more tolerant. The bioassay data

were normalized to make possible a more direct comparison of the toxic effects of whole wastes from Mills A, B and C on each species of fish. Figure 1, and Table 1 show the relative tolerance of each species of fish to each mill effluent. Means from the 96-hour normalized toxicity indicies were used in plotting figure 1.

Each species of fish displayed a general relative tolerance to whole wastes from all three mills (e.g., goldfish tested were always more tolerant than bluegills in the same waste dilutions and under the same test conditions), but each species, especially adult guppies, differed in its relative tolerance to wastes from each mill. For example, in Figure 1, the histogram shows that adult guppies tested were most tolerant of Mill C wastes and least tolerant of Mill B wastes, while just the opposite occurred with bluegills, which displayed the most tolerance for Mill B wastes and the least for Mill C wastes. Other species tested also showed a unique tolerance among the three mill wastes.

Coho salmon and bluegills were much more sensitive to Mill B wastes at 24 hours than were guppies, but at 48 or 96 hours there were only small differences in the tolerances. No such large effect is seen in wastes from Mills A and C. The complex chemical nature and rapidity of toxic action of these wastes is undoubtedly responsible for much of the variation.

Untreated whole effluent samples frequently become somewhat less toxic after a relatively short period of rapid aeration. This change can be attributed primarily to the removal of highly volatile or rapidly oxidizable toxic substances. Results of preliminary experiments on whole wastes indicated that although this toxicity was not great, it still was significant at times, and

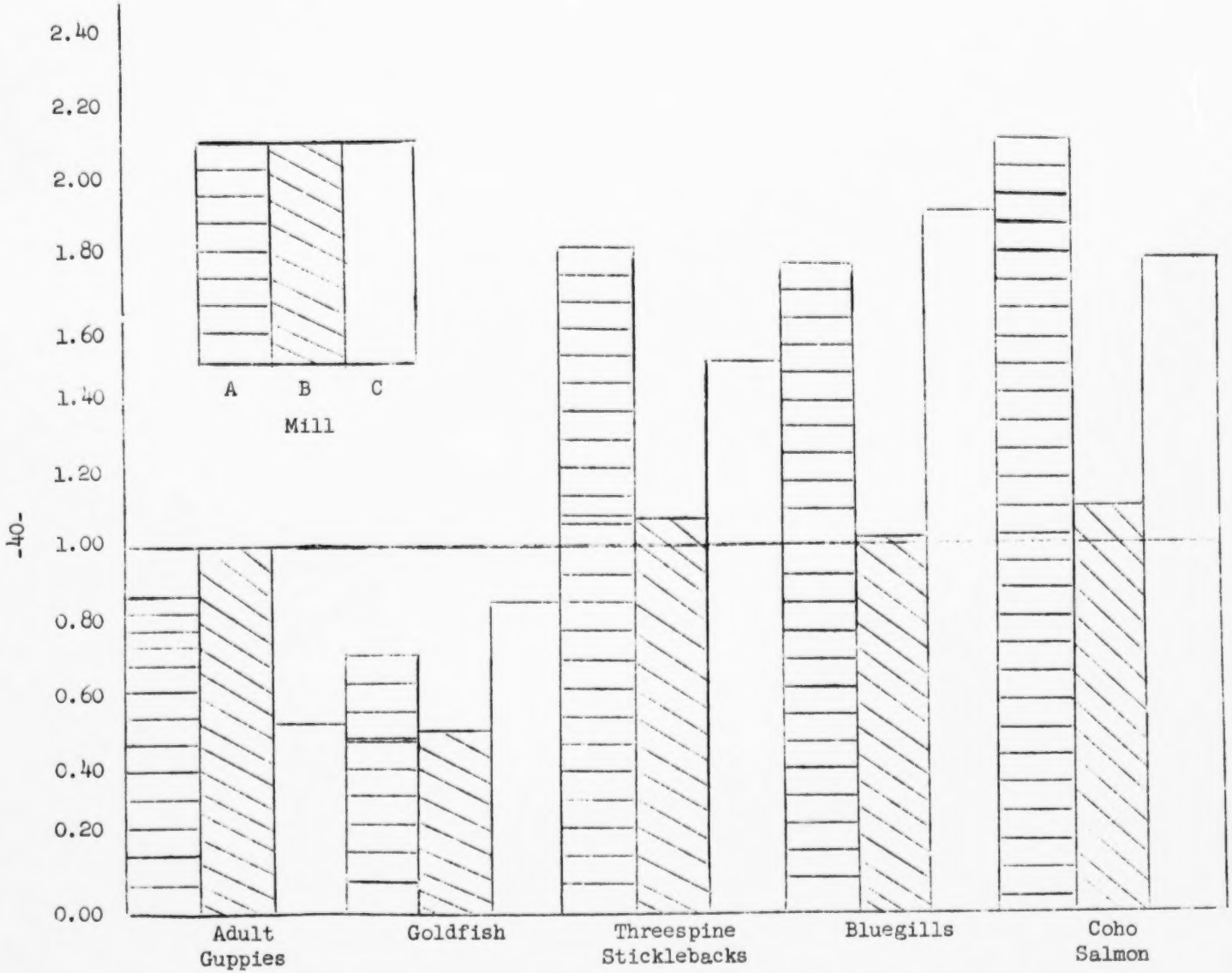

Figure 1. The relative tolerance of five species of fish to whole wastes from mills A B and C.

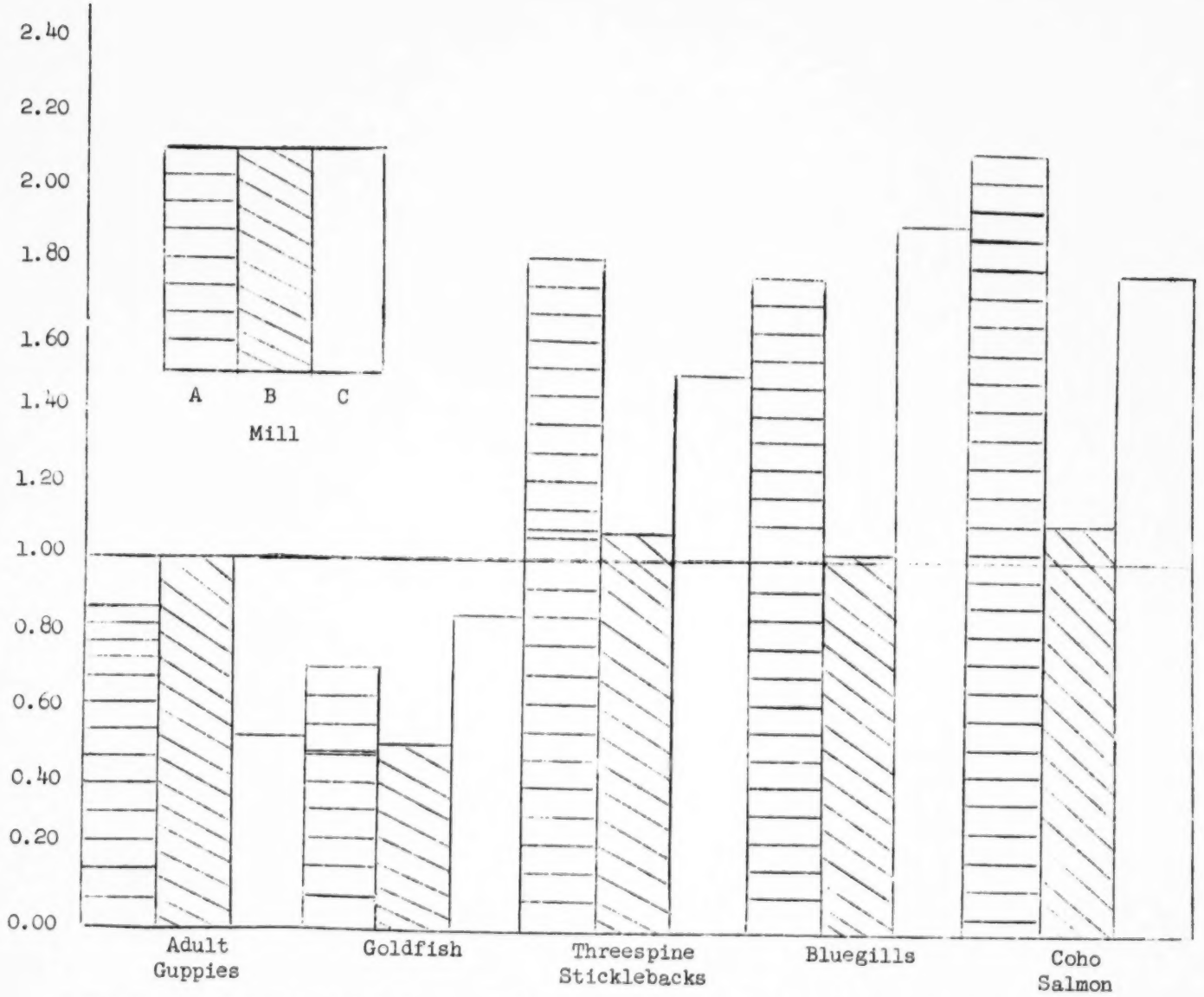

Figure 1. The relative tolerance of five species of fish to whole wastes from mills A B and C.

Table 1. Relative toxicity of whole kraft wastes from Mills A, B and C to five unrelated species of fish and the normalized toxicity index for each

Date of bioassay	Source of effluent	Exposure time in hours	TL_m of young guppies	Young guppies	Adult guppies	goldfish	Threespine stickleback	Bluegill sunfish	Coho salmon
2-28-61	Mill A	24	43	1.00	--	0.58	1.35	1.89	1.80
		48	32	1.00	--	0.76	1.00	2.48	2.37
		96	24	1.00	--	0.57	1.07	1.86	1.78
3-27-61	Mill A	24	39	1.00	0.82	0.93	2.82	1.85	2.41
		48	37	1.00	0.86	0.88	2.68	1.75	2.57
		96	35	1.00	0.86	0.83	2.54	1.66	2.43
3-15-61	Mill B	24	24	1.00	0.75	0.47	--	3.11	3.20
		48	7.5	1.00	0.75	0.61	--	1.00	1.00
		96	7.5	1.00	0.97	0.61	--	1.00	1.14
4-24-61	Mill B	24	12.4	1.00	--	0.30	2.76	2.76	2.95
		48	4.7	1.00	--	0.38	1.12	1.09	1.12
		96	4.5	1.00	--	0.36	1.07	1.05	1.07
2-22-61	Mill C	24	15.1	1.00	0.42	0.93	1.28	2.19	2.01
		48	10	1.00	0.42	0.74	1.12	1.79	1.33
		96	10	1.00	0.42	0.74	1.19	2.00	1.51
4-14-61	Mill C	24	13.5	1.00	0.59	0.94	1.53	1.75	1.80
		48	13.5	1.00	0.61	0.94	1.80	1.80	1.88
		96	13.5	1.00	0.61	0.94	1.80	1.82	2.05

could cause "unexplained" differences in bioassay results if it were not taken into consideration. The contribution of this rapidly lost toxic fraction to the overall toxicity of whole wastes was studied for Mills A, B and C.

Acute toxicity bioassays were performed on untreated wastes and on wastes which had been aerated 4 or 24 hours. Part of the bioassay solutions received no oxygenation, while duplicate solutions received slow oxygenation or normal aeration. Means of the normalized TL_m values obtained are presented in Table 2. The normalized toxicity values for the nonaerated wastes are generally higher than those of the rapidly aerated wastes, especially for Mills B and C. Oxygenation of the bioassay waste solutions from Mills B or C also appears to reduce the toxicity. Results from bioassays on Mill A wastes were more variable, and in some cases aeration of the wastes or bicassay solutions increased the toxicity.

Repeated tests have been undertaken in order better to evaluate the relative importance of the nonvolatile distillable and nondistillable toxic components of the effluents from three representative mills.

The means of the values obtained in testing different samples from each mill studied, and the means of these means are shown in Table 3. Most of these values have changed only slightly from previous progress reports, although approximately twice as many tests are represented now.

Although the normalized results obtained in testing different samples from each mill varied rather widely, the means shown in Table 2 indicate no very striking differences between the observed characteristics (other than overall toxicity) of the effluents from the three mills studied. It is apparent, however, that a larger part of the toxicity of the whole effluent

Summary of results of definitive experiments on the relative toxicity of aerated and non-aerated effluents from mills A, B, & C.

Source of effluents	Treatment of effluents	Oxygenation in Bioassays	Number of samples	Median tolerance limit			Normalized toxicity index		
				24-hr	48-hr	96-hr	24-hr	48-hr	96-hr
	None	None	8	37.8	27.2	22.8	1.38	1.75	1.36
	None	Air	13	38.6	31.6	23.3	1.34	1.40	1.30
	None	Oxygen	3	30.2	27.9	26.1	1.22	1.09	0.99
Mill A	Aerated 4 hr	None	8	54.1	38.9	34.7	1.03	1.03	1.09
	Aerated 4 hr	Air	13	49.5	34.1	27.7	1.00	1.00	1.00
	Aerated 4 hr	Oxygen	3	39.0	30.6	24.3	0.93	1.14	1.12
	Aerated 24 hr	Air	5	72.2	40.8	25.5	0.81	0.72	0.65
	None	None	9	15.6	7.9	6.4	1.40	1.58	1.81
	None	Air	19	18.2	9.0	7.8	*1.23	1.25	1.19
	None	Oxygen	3	22.8	8.2	7.2	0.96	1.31	1.45
Mill B	Aerated 4 hr	None	9	22.8	11.2	9.6	0.93	1.09	1.14
	Aerated 4 hr	Air	18	21.4	10.6	8.9	1.00	1.00	1.00
	Aerated 4 hr	Oxygen	3	29.0	12.4	11.4	0.81	0.91	1.03
	Aerated 24 hr	Air	3 or 5	31.9	8.9	6.7	0.67	1.52	1.15
	None	None	7	8.3	5.8	4.5	2.42	2.21	1.79
	None	Air	15	*13.3	8.2	6.2	*1.29	1.61	1.30
	None	Oxygen	3	7.2	5.6	5.0	2.19	1.55	1.35
Mill C	Aerated 4 hr	None	7	17.2	10.1	7.4	1.05	1.34	1.10
	Aerated 4 hr	Air	15	*17.8	11.4	7.1	1.00	1.00	1.00
	Aerated 4 hr	Oxygen	3	13.3	9.0	8.1	1.59	1.59	1.13
	Aerated 24 hr	Air	7	27.1	17.4	11.3	**0.71	0.86	0.90

from Mill A than of effluents from Mills B and C was due to distillable constituents, as a general rule, and that these constituents were least important in the effluent from Mill B.

After 24-hour exposure of the fish to the test dilutions, the distillates of all the effluents generally proved quite toxic, whereas relatively little or not toxicity of the diluted residues was demonstrable. However, the final results of the 96-hour tests indicate predominant importance of the nondistillable toxic components (residues) of most of the effluent samples, especially the samples from Mills B and C. On the basis of the 96-hour test results, the average toxicity of the distillates of Mill A effluent can be said to be nearly equal to that of the diluted residues, but the toxicities of the distillates of Mill C and Mill B effluents were much less. The normalized toxicity indices of the residues recombined with the distillates are not quite as great, on the average, as those of the effluent sample portions that had only been aerated for 4 hours, but are greater than the sums of the values shown for the separately tested distillates and residues. The sum of the mean 96-hour indices of the two separate fractions of effluent samples from all the three mills is 0.77 (0.27 + 0.50), whereas the corresponding mean value for recombined residues and distillates is 0.97. Synergistic interaction of the distillable and nondistillable waste components is indicated.

Table 3. Summary of results of definitive experiments on the relative toxicity to guppies of fractions of whole effluents from three kraft mills and their interaction.

Mill	Exposure time	Mean TL_m (percent) effluent aerated 4 hours	Effluent untreated	Effluent aerated 24 hours	Distillate	Residue	Distillate plus Residue
A	24 hours	50.6	1.47	<0.81**	0.66**	<0.51*	0.91
B	"	26.8	1.07	0.67	0.33	<0.22*	0.77
C	"	18.5	1.39	0.71	0.52	<0.31*	0.96
Mean		32.0	1.31	<0.73**	0.50**	0.35*	0.88
A	48 hours	30.0	1.41	0.72	<0.41**	<0.31*	0.74
B	"	9.6	1.07	0.77	0.17	<0.48**	1.00
C	"	11.4	1.59	0.77	0.41	0.49	1.20
Mean		17.0	1.36	0.75	<0.33**	<0.43*	0.98
A	96 hours	21.6	1.20	0.69	0.35	<0.34**	0.76
B	"	7.7	1.05	0.75	0.16	0.70	1.12
C	"	6.7	1.26	0.90	0.29	0.46	1.03
Mean		12.0	1.17	0.78	0.27	<0.50**	0.97

* True mean may be much less than the value shown, because TL_m of most or all samples was greater than 100 per cent (not determined).

** True mean value not much less, as the TL_m of only one or two of the samples being indeterminate (greater than 100 per cent).